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Contradictions in X-ray structures of intermediates in the photocycle of photoactive yellow protein

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To the Editor — In the March 2013 issue of *Nature Chemistry*, Jung *et al.*[1] reported time-resolved X-ray structures of early intermediates in the photocycle of photoactive yellow protein (PYP), a model system that has long served as a paradigm for understanding photoisomerization in proteins. In agreement with Schotte and colleagues[2], the experimental electron density map recovered by Jung *et al.* for their first intermediate, which they denote I_T, unveils a highly contorted *p*-coumaric acid (pCA) chromophore with its carbonyl group oriented nearly perpendicular to the phenolate ring (Fig. 1a,b). However, the X-ray structures assigned to I_T and a subsequent intermediate denoted I_{CT} are inconsistent with density functional theory (DFT) calculations and previously published structures. Here, we report new DFT calculations that lend additional support for the structures reported in Schotte *et al.* and help reconcile contradictions arising from the X-ray structures of I_T and I_{CT}.

The unusual pCA conformation in Fig. 1a,b begs a question regarding how this twist is accommodated stereochemically among the three dihedral angles between the phenolate and the carbonyl (Fig. 1c). Of key mechanistic importance is the central dihedral angle involving the C_3=C_2 double bond, whose *trans*-to-*cis* photoisomerization drives the structural transitions that ultimately lead to the signalling state of PYP. The 79°/81° central dihedral angle found in the Jung *et al.* X-ray structures for I_T (PDB ID: 3VE3/4I38) is in stark contrast with the 21° angle extracted from their DFT-optimized structure, and contradicts the assertion that the DFT results support their interpretation of I_T. Whereas a ~90° angle “halfway between the *trans*- and *cis*isomers” has been predicted for the electronic excited state of pCA (ref. 3), the persistence of I_T far beyond the ~2 ps excited state lifetime of PYP (ref. 4) suggests that it corresponds to a ground-state species. More plausible is their DFT structure for I_T, a strained *cis* conformation that is consistent with the Schotte *et al.* X-ray and DFT structures for the corresponding pR_0 intermediate[2] (PDB ID: 4B9O; Fig. 1d).

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Although quite different stereochemically, the X-ray and DFT structures for I_T thread through the experimental I_T electron density maps with high fidelity (Fig. 1a,b). Indeed, the pCA atomic coordinates differ by only 0.18 Å r.m.s., and when refined against 1.6 Å diffraction data, the difference in their crystallographic R factor was found to be negligible ($\Delta R_{cryst} = 0.0001$). For this comparison, PHENIX was used to refine atomic models against the structure-factor amplitudes reported for I_T (PDB ID: 4I38), for which $R_{cryst} = 0.2266$ when the three dihedral angles between the pCA phenolate and carbonyl were fixed at the values found in the X-ray structure of Jung *et al.*, versus $R_{cryst} = 0.2267$ when fixed at the corresponding values in their DFT structure. The electron density maps in Fig. 1a,b were extrapolated from low- occupancy (<10%) difference maps, and lack the spatial resolution required to refine the coordinates of individual pCA chromophore atoms without chemical restraints. During their X-ray structure refinement, Jung *et al.* gradually released the dihedral angle restraint across the $C_3{=}C_2$ bond and recovered a structure whose central dihedral angle deviated significantly from that found in their DFT structure. Using the DFT methods reported by Schotte and colleagues[2], we found the I_T X-ray structure of Jung *et al.* to be unstable: it converged to a conformation similar to their I_T DFT structure and the pR_0 structures of Schotte *et al.* (Fig. 1D).

According to the kinetic model of Jung *et al.*, I_T bifurcates into I_{CT} (PDB ID: 3VE4/4I39) and pR_1, with I_{CT} supplanting the planar *cis* intermediate reported in prior time-resolved X-ray (PDB ID: 4BBT, 1TS8)[2,5] and cryo-crystallography studies (PDB ID: 1OT9, 1UWP)[6,7]. This model implies that the well-characterized planar *cis* intermediate, denoted I_{CP} by Ihee and colleagues[5], does not exist. The opposite seems more plausible: we performed DFT calculations on I_{CT} and found that its structure was unstable, and converged to a conformation similar to the DFT structures reported for I_T and pR_0 (Fig. 1d). Jung *et al.* invoked I_{CT} to explain persistence of a twisted intermediate following the decay of I_T, whereas Schotte *et al.* accounted for this persistence with a reversible transition between pR_0 and pR_1 (in the notation of Schotte *et al.*, pR_1 is similar to the intermediate I_{CP}), a view that is supported by their similar DFT energies[2]. Had Jung *et al.* allowed for this reversibility, they could have accounted for their time-resolved electron density maps with I_{CP}, whose structure is supported by both DFT and prior crystallography studies[2,5].

How might differences in the buffer conditions influence the results? In ~2.8 M ammonium sulfate, two long-lived pR intermediates were required to account for the time-resolved diffraction data[1,5]. In 1.1 M NaCl and 2.5 M ammonium sulfate, which is arguably more physiologically relevant for halophilic bacteria, only one long-lived pR intermediate was required[2]. Whereas 1.1 M NaCl seems to simplify the PYP photocycle, it is difficult to rationalize how its absence could stabilize I_T in a high-energy twisted state, or morph planar I_{CP} into an unstable, twisted I_{CT} conformation.

In conclusion, the contradiction between Jung and colleagues' X-ray and DFT structures for I_T arises from an empirical choice to loosen rather than tighten their dihedral angle restraint across the $C_3{=}C_2$ bond. This choice seems not to be compelled by their diffraction data, and results in an X-ray structure that is more consistent with the electronic excited state. If we make the more plausible assumption that I_T is a ground-state intermediate, the unveiling of "… a long-hypothesized highly twisted intermediate along the *trans*-to-*cis* isomerization pathway" has not yet occurred, but may prove possible with a free-electron X-ray laser such as the Linac Coherent Light Source, where sub-ps time resolution can be achieved.

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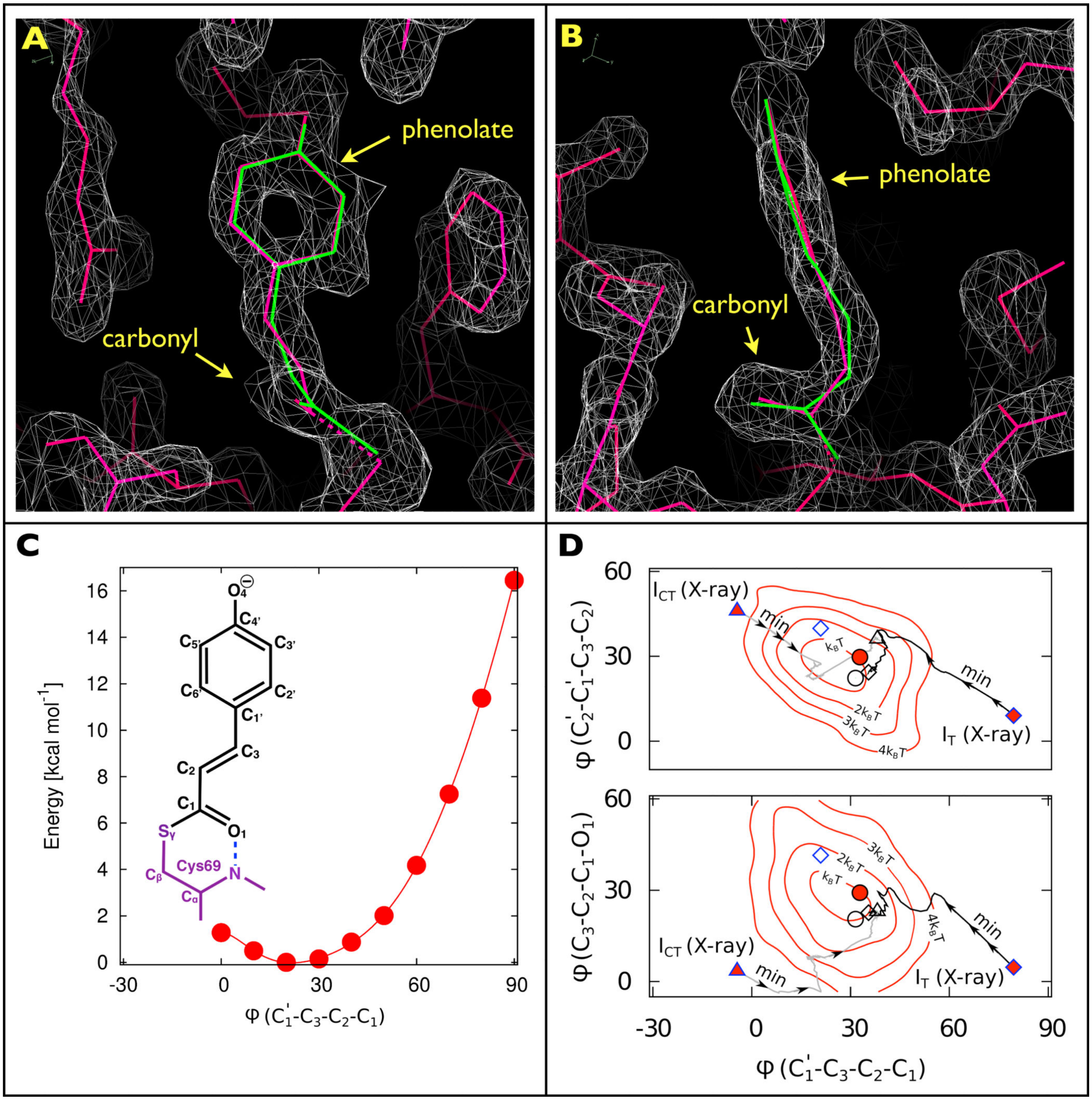

Figure 1.

Structures and stereochemistry of early intermediates following photoactivation of PYP. **a**, Front and **b**, side views of the pCA chromophore and its immediate surroundings. The two electron density maps for I_T (grey), contoured at 1.64σ, clearly show the carbonyl oriented perpendicular to the phenolate plane. Although quite different stereochemically, both X-ray (red) and DFT (green) pCA structures thread through the electron density maps with high fidelity. **c**, Labelling scheme for the pCA chromophore and DFT energies (calculated at the D-B3LYP/def2-TZVP level) for the C_3=C_2 dihedral angle (φ) of pR_0. **d**, Dihedral angles of early intermediates reported by Jung *et al.* (blue outline: I_T as diamonds; I_{CT} as triangles), and Schotte *et al.* (black outline: pR_0 as circles), for both X-ray refined structures (red-filled symbols) and DFT-optimized structures (open symbols). The X-ray structures for I_T and I_{CT} were found to be unstable: during DFT structure optimization, both converged to structures similar to pR_0 (the black and grey lines, labelled 'Min', indicate the dihedral projections along the energy minimization pathways obtained during the respective structure optimizations). The DFT calculations reported in Schotte *et al.* included 176 atoms (D-BP86/def2-SVP); Jung *et al.* included 157 atoms (B97-1/6-31G(d)/3-21G). Underlaid are dihedral/dihedral free-energy contours computed from a 5 ps hybrid quantum/classical mechanics (QM/MM) simulation[2,8] of the pR_0 intermediate with 143 QM atoms/2,171 MM atoms (simulations were performed using CHARMM/Q-Chem: D-BP86/def2-SVP/ CHARMM27). Note that in this projection, the Jung *et al.* DFT structure for I_T differs from the I_T, I_{CT} and pR_0 DFT-optimized cluster by only ~1 kT in free energy.